

Rapid Detection of Sublethally Impaired Cells of *Enterobacteriaceae* in Dried Foods

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Samples of dried foods, feeds, and drugs were examined for *Enterobacteriaceae* by using buffered glucose-Brilliant Green-bile broth as the final enrichment medium and deep tubes of violet-red-bile-glucose-agar for confirmation. The pre-enrichment treatments used for the resuscitation of sublethally impaired cells were overnight incubation in lactose broth versus incubation for 1 to 6 hr at room temperature in shallow layers of tryptone soya peptone broth. The latter restoration treatment appeared to be significantly more productive.

It is, at present, well established that *Enterobacteriaceae*, occurring in dried foods and feeds, may carry metabolic lesions that impair the physiology of the cells to the extent that they will not proliferate in the customarily used selective media containing inhibitors in concentrations that are well tolerated by nonimpaired cells of the same species or serotype (1, 11). Such sublethal lesions stem from thermal stressing, sojourn in an environment of very low water activity, oxygenation, or a combination of such factors (12). Cells, physiologically injured in this way, will recover in foods when the water activity allows their proliferation. Attempts should, therefore, always be made also to detect such injured cells when examining dried foods for pathogenic or index organisms. This requires submitting stressed cells to a restoration treatment prior to their exposure to selective media currently used for their detection or enumeration. Such a step is generally indicated as "resuscitation" of debilitated cells (2).

In the microbiological examination of dried foods, overnight incubation of suitable samples of foods in lactose broth is generally employed for the purpose of resuscitation (4, 9, 10). However, more recent investigations on the physiology of sublethally stressed cells have demonstrated that restoration of gram-negative rod-shaped bacteria may be complete within the order of a few hours at temperatures in the range of 20 to 30 C, provided the right external conditions are chosen (1, 5, 11). Such a rapid technique would lead to having the results available at least 1 day ahead of those obtained by the standard procedure. Also, it would avoid prolific growth of other bacteria in the resuscitation

medium and hence suppression of *Enterobacteriaceae* by the latter (9).

We have, therefore, investigated whether a brief incubation of suspensions of dried foods in a nutritionally rich fluid, e.g., tryptone soya broth, under aerobic conditions would be as effective in resuscitating stressed cells as the usual overnight incubation in lactose broth.

A total of 167 samples of dried foods, drugs, and feeds were examined. The commodities were chosen from those that have presented health risks in the past (9). Approximately the same relative numbers of samples of a given commodity as used in our previous investigations (9) were examined. An inventory of the samples is presented in Table 1.

Buffered tryptone soya peptone broth (TSB; Oxoid Ltd., London), found in other investigations to be a most effective resuscitation medium (3), was used as the suspension fluid; it contained tryptic digest of casein, 17 g; papaic digest of soya protein, 3 g; glucose, 2.5 g; NaCl, 5 g; K₂HPO₄, 2.5 g; distilled water, 1,000 ml. Resuscitation was effected by incubating 1:10 dilutions of the foods under investigation in this broth. One milliliter of each diluted food was transferred to a standard culture tube, 10 ml of each to a 200-ml Erlenmeyer flask, and 100 ml to a 500-ml Erlenmeyer flask. In each case, the resulting fluid layers were not much deeper than ca. 1 cm. Incubation was carried out at 19 to 25 C for 1 to 6 hr, samples being examined every hour.

Standard resuscitation was done by the current United States practice. Samples of the dry foods were cultured overnight in 10-fold volumes of lactose broth at 30 C (10).

As the control test without resuscitation, dilu-

TABLE 1. Comparison of the productivity of two resuscitation methods in the examination of dried foods for *Enterobacteriaceae*

Type of food	No. of samples (0.1 g) examined							Frequency distribution of time required for resuscitation (hr) at room temp (21 to 24 C) in TSB ^a		
	Total examined	Negative, all methods	Negative, standard resuscitation method	Positive, standard resuscitation method	Positive, without resuscitation	Positive, upon resuscitation, but negative without resuscitation		1	2	6
						Standard technique	New technique			
Animal feeds.....	12	6	7	5	4	1	2	2		
Cocoa.....	8	5	5	3	3	0	0			
Drugs.....	6	6	6	0	0	0	0			
Eggs, dried.....	13	7	7	6	5	1	1	1		
Infant formula, dried.	40	24	29	11	9	2	5	3	1	1
Milk, dried.....	31	28	31	0	1	0	2	1		1
Noodles.....	8	5	6	2	0	2	3	2	1	
Soups, dried.....	49	9	10	39	27	12	13	9	4	
Totals.....	167	90	101	66	49	18	26	18	6	2

^a Readings were taken at hourly intervals from 1 to 6 hr. There were no new positive samples at 3, 4, and 5 hr.

tions were planted immediately after preparation in 10-fold volumes of buffered Brilliant Green-bile-glucose (EE) broth (8).

To all previously resuscitated suspensions, 10-fold volumes of EE broth were added after the period of time taken for resuscitation. EE tubes and flasks were incubated overnight at 30 C and thereupon read for turbidity, gas formation, and brilliant green reduction. The enrichment cultures so obtained were stabbed in freshly steamed deep tubes (6) of violet-red-bile-glucose agar (7) and incubated at 30 C. After both 8 hr and overnight incubation, the tubes were inspected for the typical growth pattern of *Enterobacteriaceae*, i.e., development of an inoculation line reaching to the bottom of the tube surrounded by a coaxial cylinder of purple precipitate. Positive cultures were examined for oxidase activity to preclude their being *Aeromonas* species (7).

The gross results obtained are also summarized in Table 1. With regard to the efficacy of the various procedures applied, the following can be concluded.

The need for some sort of resuscitation is demonstrated once more by our data. The number of true *Enterobacteriaceae*-positive samples amounts to 77, i.e., the difference between the totals of columns 3 and 2 of Table 1. Without any resuscitation treatment, only 49 positives would have been found, which means that slightly under 40% of the positives would have been missed by immediate planting of the food dilutions in a selective medium.

In no instance did the new, more rapid, resus-

citation technique lead to falsely negative results for *Enterobacteriaceae*, in comparison to the customarily used standard procedure. The inverse was observed in eight instances (see difference between positives in columns 8 and 7 of Table 1), or over 10% of the 77 positive samples. This is most probably due to other bacteria outgrowing the *Enterobacteriaceae* in the course of the long overnight incubation in the nonselective lactose broth used in the standard technique (9).

The optimal duration of exposure to the new resuscitation technique used appeared to be 1 to 2 hr. Apparently, a longer restoration treatment under these conditions will also lead to partial overcrowding of the medium by other bacteria. This period of time of ≤ 2 hr required for resuscitation of gram-negative rods agrees rather well with the procedures found optimal empirically in fundamental studies in this area (1, 5, 11).

These results also make it likely that, in customarily used procedures for the enumeration of *Enterobacteriaceae* in dried foods, enough time elapses and sufficient aeration is provided during the preparation of decimal dilutions to allow a reasonable degree of restoration of stressed cells. These cells will then be detected with the part of the population not affected by the stress, growing in the selective EE medium without any resuscitation treatment.

LITERATURE CITED

- Alford, J. A., and N. L. Knight. 1969. Applicability of aeration and delayed addition of selenite to the isolation of *Salmonellae*. *Appl. Microbiol.* 18:1060-1064.
- Allen, L. A., S. M. Pasley, and M. S. F. Pierce. 1952. Conditions affecting the growth of *Bacterium coli* on bile salts

- media. Enumeration of this organism in polluted waters. *J. Gen. Microbiol.* 7:257-267.
3. Dabbah, R., W. A. Moats, and J. F. Mattick. 1969. Factors affecting resistance to heat and recovery of heat-injured bacteria. *J. Dairy Sci.* 52:608-614.
 4. Levine, M., R. H. Tanimoto, H. Minette, J. Arakaki, and G. B. Fernandes. 1955. Simultaneous determination of coliform and *Escherichia coli* indices. *Appl. Microbiol.* 3:310-314.
 5. Lewicki, P. P., and G. J. Silverman. 1969. Mechanisms concerned in the repair and recovery of sublethally impaired cells occurring in foods, p. 106-134. *In* The microbiology of dried foods. Proc. 6th Int. Symp. Food Microbiol., Haarlem.
 6. Mossel, D. A. A., and G. Martin. 1961. Milieu simplifié permettant l'étude des divers modes d'action des bactéries sur les hydrates de carbone. *Ann. Inst. Pasteur Lille* 12:225-226.
 7. Mossel, D. A. A., W. H. J. Mengerink, and H. H. Scholts. 1962. Use of a modified MacConkey agar medium for the selective growth and enumeration of *Enterobacteriaceae*. *J. Bacteriol.* 84:381.
 8. Mossel, D. A. A., M. Visser, and A. M. R. Cornelissen. 1963. The examination of foods for *Enterobacteriaceae* using a test of the type generally adopted for the detection of *Salmonellae*. *J. Appl. Bacteriol.* 26:444-452.
 9. Mossel, D. A. A., and H. Vincentie. 1969. Ecological studies on the enrichment of *Enterobacteriaceae* occurring in dried foods in some currently used media, p. 135-159. *In* The microbiology of dried foods. Proc. 6th Int. Symp. Food Microbiol., Haarlem.
 10. North, W. R., Jr. 1961. Lactose pre-enrichment method for isolation of *Salmonella* from dried egg albumen. Its use in a survey of commercially produced albumen. *Appl. Microbiol.* 9:188-195.
 11. Sinskey, T. J., and G. J. Silverman. 1970. Characterization of injury incurred by *Escherichia coli* upon freeze-drying. *J. Bacteriol.* 101:429-437.
 12. Webb, S. J. 1969. The effects of oxygen on the possible repair of dehydration damage by *Escherichia coli*. *J. Gen. Microbiol.* 58:317-326.